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Attenuated immunogenic parasites are essential in the transfer of immunity to virulent *Plasmodium berghei*

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SUMMARY

A less virulent parasite of *Plasmodium berghei* K173 was isolated that induced immunity against the more virulent parasite. Immunity to this parasite but not to the virulent one, could be transferred by immune spleen cells but not by immune lymph node cells. However, the immune spleen cells did transfer immunity to the virulent strain if accompanied by infection with viable parasites of the less virulent strain, but only if they were allowed to proliferate for a period of 1 week before challenge with the virulent strain. Immune spleen cells could survive two cycles of mouse to mouse transfer. The induction of immunity by transfer of immune spleen cells was associated with the production of anti-parasitic antibody.

INTRODUCTION

To induce immunity against the malaria parasite *Plasmodium*, several immunization procedures have been employed using parasites with self-resolving infections[1–3] or parasites that have been administered under chemotherapeutic control,[4–8] to allow the parasite to be maintained at appropriate levels capable of stimulating the immune system without totally overwhelming it.

One such immunization procedure employed in this laboratory involved the injection of $1–3 \times 10^7$ virulent *Plasmodium berghei* (K173) parasites and the application of sulphonamides for 5 weeks.[7] After this time, mice were able to control a new challenge with the parasite. It appeared that the persistence of the live parasite during the 5-week immunization period was important for development of immunity.[6,7,9–11] In addition, immunosuppressive measures preventing induction of immunity were particularly effective in the last week of the 5-week immunization period.[12,13] This suggested that during the last part of the immunization period specific changes occurred in the host–parasite interaction. By transfer of spleen cells or blood of mice that were immunized by the 5-week immunization procedure to naive recipient mice, it appeared that the persisting parasites in immunized mice were attenuated (manuscript submitted for publication). In normal mice these parasites did not induce cerebral malaria, while the virulent parent strain did.[14] Transient infections were observed when C57Bl and B10LP mice were inoculated in low numbers ($< 10^3$ parasitized red blood cells/mouse), and under chemotherapeutic control mice could be immunized within a 3-week period. The less virulent, attenuated parasites appeared to be essential for the induction of immunity in mice that received spleen cells from immune donor animals. Because of these characteristics, this type of parasite was called immunogenic (IP) in comparison to the original, more virulent, wild type (VP).

A variety of transfer models (e.g. rat and mouse) using parasites with self-limiting infections, attenuated parasites or parasites that are virulent in some, but not all, hosts has been reported.[15–17] A role for attenuated parasites was not established in any of these studies. However, the finding by Fahey & Spitalny[15] that 7 and 14 days, but not 21 and 28 days, after an immunizing infection with *P. yoelii*, immunity could be transferred by spleen cells, is compatible with a role for the parasite in an immunizing inoculum.

This paper reports on the immunization of mice by the inoculation of spleen cells from *P. berghei*-immune donors, provided that the recipients were infected with IP. Transfer of immune spleen cells protects against IP but not VP challenge, and stimulation of these immune spleen cells by live, proliferating IP, was essential for induction of immunity to the VP in recipient mice. It is shown that transferred immune spleen cells persisted in the recipient mice. In addition, development of anti-parasitic antibody appeared to be related to the development of immunity in the mice that received immune spleen cells, were boosted with IP and resisted challenge with VP.

MATERIALS AND METHODS

Mice

Naive, male and female C57BL/10 or C57BL/6 J mice, used as recipients at 6–8 weeks old, were obtained from the central

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animal facility at the University of Nijmegen (the Netherlands). They were housed under specific pathogen-free conditions in plastic cages (39 × 22 × 14 cm) and received food (RMH, Hope Farms, Woerden, the Netherlands) and water *ad libitum*. All mice in any particular experiment were age-matched.

Parasites

Both the original wild type and an immunogenic form derived from the wild type of *P. berghei* K173 were used in these studies. The development of the immunogenic form has been described in detail elsewhere (manuscript submitted for publication). Briefly, IP were isolated on day 28 of a 5-week immunization procedure by the subinoculation of blood of mice being immunized by a chemotherapeutically controlled infection (see below) into splenectomized mice.

VP were kept viable by weekly passage in intact mice, and IP by weekly or biweekly passage in splenectomized C57BL/10 mice using 10^5 parasitized red blood cells obtained via the tail blood of infected mice. Tail blood was collected in sterile, physiological saline containing 5 U heparin/ml, adjusted to the appropriate concentration by determination of the percentage parasitaemia, and injected intraperitoneally (i.p.) into passage or experimental mice. The percentage parasitaemia was determined by counting parasitized red blood cells and uninfected red blood cells appearing in May–Grünwald–Giemsa (Merck, Darmstadt, Germany)-stained thin blood smears.

Chemotherapeutic control of parasite infections

Chloroquine treatment consisted of 100 mg chloroquine base/l in the drinking water for a period of 5 days, and was given to cure an infection radically or to eliminate persisting parasites from immune mice.

Sulphonamide-treated mice received either 150–300 mg sulphathiazole Na/l or 20–30 mg sulphadiazine/l in their drinking water to suppress an infection to subpatent levels. Chemotherapy was stopped 2 days before a subsequent infection or transfer of cells.

Immunization of mice

Immunization of mice to VP by a chemotherapeutically controlled 5-week infection has been described elsewhere.[7] Briefly, mice were given $1–3 \times 10^7$ parasitized red blood cells. To prevent clinical malaria, suppressive treatment was started 2 days after injection by the addition of sulphonamide to the drinking water for 33 days. Two days after withdrawal of the sulphonamide treatment, the mice were challenged with 10^5 VP to ascertain immunity. Mice were judged immune if parasitaemia did not go above 1% or stayed at or returned to subpatent levels. Mice with established immunity are usually carriers of subpatent numbers of parasites, which is called premunity.[6,10]

Adoptive transfer studies

A schematic representation of the manipulations performed in the final adoption of the transfer model is given in Fig. 1.

A basic format of the finally adopted transfer model is given in Fig. 1b. Briefly, $1–4 \times 10^7$ spleen cells from immune donors cleared of parasites with chloroquine, were transferred intravenously (i.v.) to recipient mice along with IP on the same day. Seven days after transfer/boosting with IP, a challenge infection was given using 10^5 VP/mouse. Blood smears were made on the day of challenge with VP and at least once a week thereafter, for a minimum period of 4 weeks after the VP challenge, when mice exhibited subpatent infections, low transient infections or death. Spleens of immune donors were pooled to obtain enough of a homogeneous preparation for injection to all experimental mice.

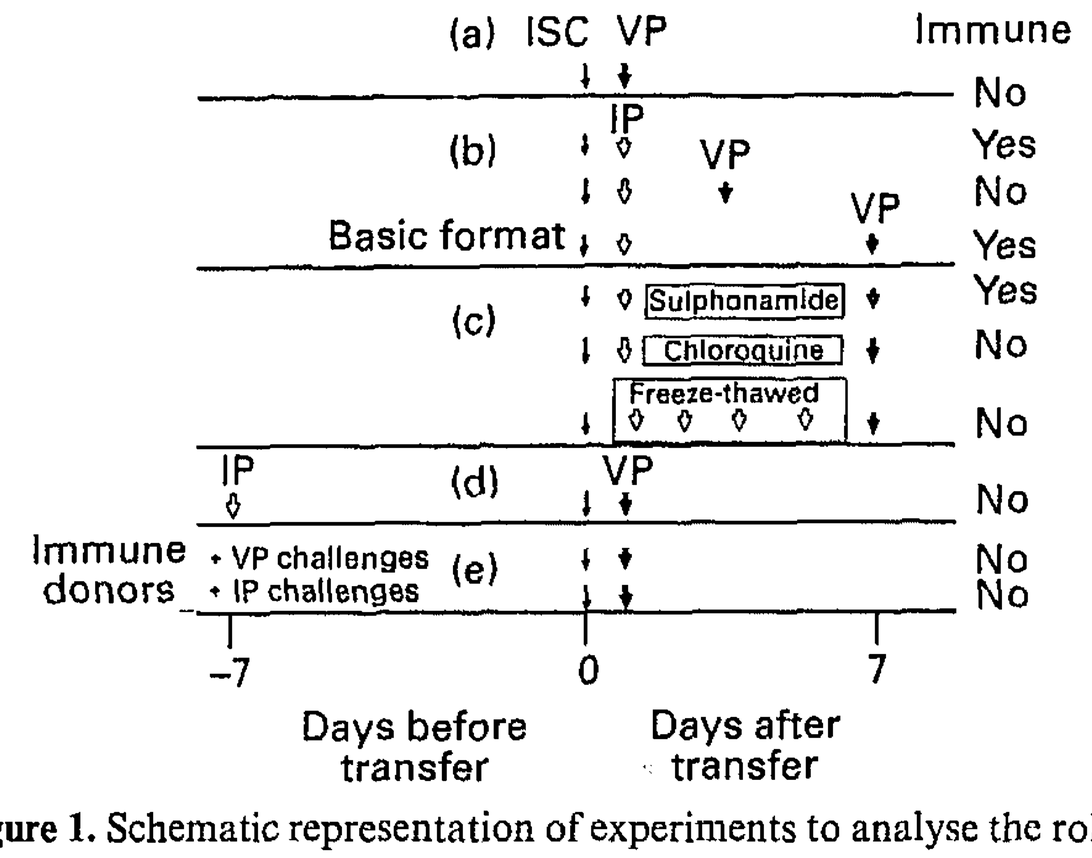

Figure 1. Schematic representation of experiments to analyse the role of transferred immune spleen cells (ISC) and boosting by viable, proliferating, immunogenic parasites (IP) in the development of immunity to challenge by the virulent parent strain (VP). In the experiments described in (a–c), donor immune mice with an established VP immunity were chloroquine treated to eliminate subpatent parasitaemias immediately before collection of the spleen cells, as described in the Materials and Methods.

Spleens and/or lymph nodes (where indicated) were removed from premune mice or from immune mice cleared of parasites after chloroquine treatment. The organs were dissociated by gentle pressure with a glass rod and passed through a sieve fitted in a 10-ml syringe containing a plug of cotton wool. Cells were counted in a 0·2% trypan blue/physiological saline solution (diluted 1:4) on a haemocytometer (Bürker-Türk, W. Schreck, Hofheim, Germany). Red blood cells were removed from the spleen cell suspensions by treatment with ammonium chloride–Tris.[18] Cell suspensions were injected i.v. via the tail.

Mice were given immune spleen cells and IP on day 0. To test if and when mice had developed immunity, a challenge with the VP was given 5, 7, 9 or 14 days later. From these experiments, the day of challenge with the VP in the final model was determined (Fig. 1b).

To determine the importance of viable, proliferating IP in the development of VP immunity by transferred immune spleen cells, disrupted (freezing and thawing) parasites were injected, or IP infections were either left untreated, suppressed or eliminated by sulphonamide or chloroquine treatment (Fig. 1c).

To determine if IP could stimulate protective cells in the recipients of immune spleen cells, mice were infected with IP, left untreated or given sulphonamide treatment, and 7 or 13 days later received immune spleen cells and a challenge with VP (Fig. 1d). Control groups were treated according to the basic format.

To determine the effect of boosting of immune donors, immune mice received one or more challenges with 10^8 IP or VP during the 2-week period before transfer of their spleen cells.

The recipients of these spleen cells were subsequently challenged with VP (Fig. 1e).

Across sex transfers

Male donor immune spleen cells are rejected by female recipients because of the presence of the H-Y antigen on male cells.[19] Using this information, immune spleen cells from either male or female donors were transferred to both male and female recipients, boosted with IP and challenged with VP according to the basic format. Immune spleen cells of recipients with an established VP immunity were collected approximately 1 month after the first transfer and given to naive male and female recipients, again using the basic format. In this way, up to four subsequent transfers were performed.

Malaria-specific enzyme-linked immunosorbent assay (ELISA)

Immunoglobulin titres to VP antigens were measured from plasma samples 5, 7, 10 and 14 days after infection with IP or VP or in recipients of immune spleen cells boosted with IP and challenged with VP 7 days later. Briefly, 96-well, flat-bottomed plates were coated with a parasite extract (100 μl/well) prepared by the lysis of 10^7 VP/ml with 1% Triton X-100 in phosphate-buffered saline (PBS). Sodium azide (0·05%) was added to prevent bacterial contamination and the plates were stored at 4° until use. Directly before use, plates were emptied, coated with 1% bovine serum albumin (CBSA)–PBS/0·1% Tween-20 buffer (buffer A) at 100 μl/well and left for 1 hr. All operations were performed at room temperature. Plates were then emptied and twofold dilutions (100 μl/well) of the test plasma or control sera (prepared from pooled immune mouse sera; IMS) were made in buffer A. Plates were incubated for 1 hr then washed with PBS, and incubated with 100 μl/well of rabbit anti-mouse immunoglobulin peroxidase (Dakopatts, Glostrup, Denmark), diluted 1 : 1000 in buffer A for 1 hr, washed with PBS and then incubated with tetramethylbenzidine and degassed for 20 min before use. The reaction was stopped with 4 N H_2SO_4 and the extinction coefficient read at 450 nm on a Titertek Multiskan MCC/340, MKII reader (I.C.N. Biochemical, Zoetermeer, the Netherlands). The titres of sera from mice with an established VP immunity were in the range of 1/1000 to 1/10 000. IMS was used as a positive reference sample in all determinations. Antibody titres in test samples are reported as a percentage of the IMS values.

RESULTS

The results of typical experiments carried out to develop the model of induction of immunity in naive recipient mice by transfer of spleen cells from immune donors are summarized in Tables 1 to 3, and a schematic representation of these experiments and their outcome is given in Fig. 1. Results of repeated experiments were summarized. This was also done when small differences in the experimental set-up had no effect on the outcome of the experiment.

Transfer of immunity by immune spleen cells

Spleen cells from premune mice, immunized by a chemotherapeutically controlled VP infection over a period of 5 weeks, were transferred to naive recipients. Although premune mice controlled their subpatent infection, the recipients of spleen cells from premune mice developed rapidly increasing infections and died. Neither control of the parasites in the transferred immune spleen cells nor control of additionally injected VP in recipients by sulphonamide treatment for a period of 1 week resulted in immunity to a subsequent VP challenge (results not shown). When the parasites in the premune mice were given to normal mice by subinoculation of blood, parasitaemia developed similarly to that observed in mice infected only with the VP.

Because of these observations, in subsequent experiments premune mice were cured radically by chloroquine treatment before their spleen cells were used for transfer. When the spleen cells from such immune donors were transferred to recipient mice and these were challenged with VP directly or 7 days later, no protection was observed, even when low amounts of VP were given (Table 1 and Fig. 1a). Again, a 1-week infection with VP controlled by sulphonamide treatment of the recipients did not result in immunity to VP challenge. Not even all the spleen cells (the equivalent of one immune spleen) from one immune mouse given to one recipient mouse was able to protect the recipient against a VP challenge (results not shown).

When immune donors received additional challenges with either VP or IP in an attempt to boost the donors before transfer of their immune spleen cells, no protection to VP challenge was obtained in the recipients (Fig. 1e; results not shown). These results suggested that a direct transfer of immunity to VP by immune spleen cells from mice with an established immunity was not possible.

Immunity after transfer of immune spleen cells and boosting with IP

When immune spleen cells from immune donor mice, cured of their subpatent infection by chloroquine treatment before transfer, were given to naive recipients, these recipients controlled a challenge with IP given immediately after the transfer of the spleen cells (Table 1 and Fig. 1b). In addition, such mice were immune to VP given 7 days after boosting with IP (Table 1 and Fig. 1b), whether or not they received a suppressive treatment with sulphadiazine during the 7-day IP stimulation period. Recipients of transferred spleen cells infected with IP developed immunity, even when VP were present during the 1-week stimulation period, as long as the VP proliferation was controlled by sulphonamide treatment (Table 1 and Fig. 1c). These results showed that IP immunity could be transferred by immune spleen cells and that boosting of recipients by IP generated protection to VP. Immunization by transfer of lymph node cells from immune donor animals and boosting in the recipient with IP was never successful; however, transfer of spleen cells from the same mice could successfully induce immunity in recipients. In addition, transfer of normal spleen cells and IP boosting in the recipients, as well as infection with IP alone in the absence of any transferred cells, was unable to protect recipient mice when they were challenged with VP 7 days later (results not shown).

Persistence of IP for induction of immunity by transfer of immune spleen cells

Observing that IP boosting of immune spleen cells in recipients generated protection to VP, the role of IP was analysed further.

Table 1. Protection of recipients of ISC to challenge with the virulent *P. berghei* parasite

Stimulation of ISC recipients (day 0)	Treatment of recipients between transfer of ISC and VP challenge (day 0–7)	VP challenge (day 7)	No. mice immune
None	None	Yes	0/6
10^2 VP	None	No	0/6
10^5 VP	Sulphadiazine	Yes	0/7
10^4 or 10^6 IP	None	No	6/6
10^4 or 10^6 IP	None	Yes	14/14
10^5 IP	Sulphadiazine	Yes	8/8
10^5 IP and 10^5 VP	Sulphadiazine	Yes	16/16
10^8 VP (freeze-thawed)	None	Yes	0/3
10^8 IP (freeze-thawed) on days 0, 2, 4 and 6	None	Yes	0/6
10^8 IP	Chloroquine	Yes	0/6

Boosting of recipients of immune spleen cells with freeze-thawed VP or IP, even with the equivalent of up to 10^8 VP or IP/mouse every other day after transfer of immune spleen cells, was not able to generate VP immunity (Table 1 and Fig. 1c). In addition, neither boosting of recipients with 10^8 IP, the proliferation of which was blocked by chloroquine treatment starting on the day of infection (Table 1 and Fig. 1c), nor an infection with 10^5 IP, which was abrogated by chloroquine treatment within 6 days, resulted in immune mice (results not shown). Thus, on the one hand untreated low-level IP infections or sulphonamide treatment of the IP infection (which suppresses proliferation but does not eliminate the parasites)[6,10] permits the development of immunity in recipients of immune spleen cells, while on the other hand chloroquine treatment of the IP infection (which eliminates the parasite) or injection of freeze-thawed IP does not. This suggested a need for live IP for the boosting of immune spleen cells after transfer, and this was analysed further.

In a further series of experiments, immune spleen cells were transferred to recipients, and at several time-points after boosting with IP a VP challenge was given. When IP infection periods of 1 week or longer were used, immunity to VP developed (Table 2 and Fig. 1b). From these data the basic format of transfer of immunity by spleen cells using IP infection/stimulation of recipients for a period of 7 days followed by VP challenge was derived (Fig. 1c).

As IP boosting of recipients of immune spleen cells was essential, the question was raized as to whether IP infection alone or before the transfer of immune spleen cells prepares for the development of VP immunity. The results of experiments described in Table 3 and Fig. 1d show that infection of recipients before transfer of immune spleen cells was not effective, and this was independent of sulphonamide control of that IP infection, while controls using the basic format became immune.

Results indicated an essential role of immune spleen cells and an infection with proliferating IP during a period of 7 days in the generation of immunity to VP. Consequently, the recipients of immune spleen cells were boosted with IP for a period of 7 days, followed by VP challenge to measure immunity.

Table 2. Effect of IP proliferation period on immunity to VP challenge in recipients of immune spleen cells

Transfer inoculum	IP proliferation period	No. mice immune/VP challenged
ISC + 10^5 IP	5 days	0/3
ISC	7 days	23/26
ISC	9 days	5/5
ISC	14 days	5/5

The effect of the number of immune spleen cells and IP in the transfer model

Transfers using varying amounts of both immune spleen cells and IP in the recipients were carried out. Figure 2 shows the summarized data (six to 69 mice per point) from a group of transfer experiments using 5×10^6–1×10^8 immune spleen cells/mouse and 10^3–10^6 IP/mouse. The results reflected the percentage of mice immune to VP. It was observed that this increased when the number of transferred immune spleen cells was greater than 1×10^7. In addition, when more IP were used (i.e. 10^4, 10^5 or 10^6), success rates increased to 100%, depending on the number of immune spleen cells. Transfers using 1×10^8 immune spleen cells were the most successful, reaching high (92–100%) success rates with 10^4, 10^5 and 10^6 IP.

In Fig. 2, summarized data combining successful and less successful transfer experiments with the same number of immune spleen cells and IP are depicted. Thus, most immune mice were seen from pooled data where 1×10^8 immune spleen cells were used, but in individual experiments fewer immune spleen cells could also repeatedly protect 100% of the mice. For practical reasons, the use of 2–4×10^7 immune spleen cells and 10^5 IP was adopted for subsequent transfer experiments.

The course of infection in recipient mice in relation to transfer of immune spleen cells and IP boosting

Usually IP infections, either given alone or after transfer of

Table 3. The effect of IP infection before or on the day of transfer of ISC on immunity to VP challenge

Day of IP infection of recipients of ISC	Treatment between transfer of ISC (day 0) and VP challenge (day 7)	No. mice immune/VP challenged
IP infection before transfer		
Day −13	None	0/3
Day −13	Sulphonamide	0/3
Day −7	None	0/6
Day −7	Sulphonamide	0/6
IP infection on day of transfer		
IP on day 0	None	5/5
IP on day 0	Sulphonamide	5/5
Controls		
IP only, no ISC	None	0/6

immune spleen cells, remained low (< 1%) during the week before VP challenge. In the absence of immune spleen cells, IP infections continue to rise and animals died 4–6 weeks later. Mice given VP 7 days after IP developed a rapidly increasing parasitaemia and died. Mice receiving immune spleen cells, boosted with IP and challenged with VP 7 days later, frequently exhibited low-grade infections (< 1%) throughout; however, patency could be observed and occasionally infections reached as high as 10% parasitaemia. In the majority of cases with elevated parasitaemias, however, infections returned to subpatent levels between 7 and 14 days after challenge with VP.

Anti-parasitic antibody and VP immunity after transfer of immune spleen cells boosted with IP and challenged with VP

Anti-parasitic antibody titres in mice receiving IP, or VP, or IP followed by VP 7 days later, remained very low, with a maximum of 10% of the IMS value observed in VP-immune mice. In recipients of immune spleen cells boosted with IP and challenged with VP 7 days later, antibody titres remained low (< 5% IMS value) until 7 days after transfer, and then increased to 15% of the IMS value on day 10 and 80% of the IMS value on day 14 in VP-immune mice. Thus, increased anti-parasitic antibody levels were only observed in immune mice.

Immune spleen cells transferred from donors to same-sex or across-sex recipient mice.

The results of same-sex and across-sex transfers using the basic format showed that immunity was obtained when male immune spleen cells were transferred to same-sex recipients, or female immune spleen cells to male recipients, but transfers of male immune spleen cells to female recipients were unsuccssesful, as expected (Table 4).

To analyse further the survival and function of transferred immune spleen cells, female immune spleen cells were transferred to males, boosted with IP and challenged with VP. Using the same protocol, immune spleen cells of these males were transferred to males again, and finally immune spleen cells from the second-series males were transferred back to females. In all of the subsequent steps, immune mice were obtained and, as shown in Table 4, the final transfer of immune

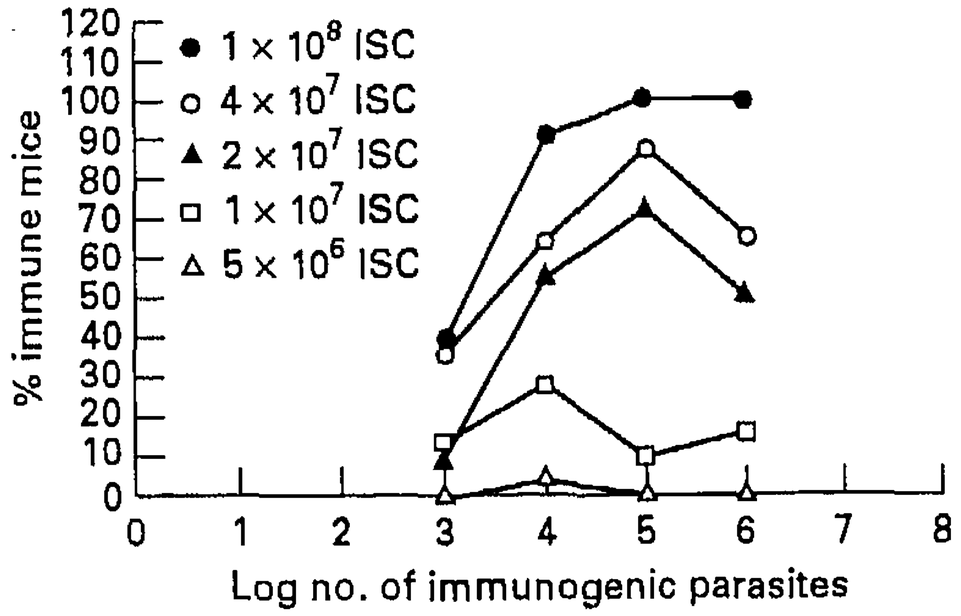

Figure 2. The percentage of mice immune to a VP challenge after transfer of different numbers of ISC and boosting with increasing numbers of IP for a period of 7 days.

Table 4. The ability of immune spleen cells to transfer immunity to same-sex or across-sex recipient mice

Type of transfer	No. mice immune/VP challenged
C57Bl/6J	
Male–Male	7/9
Male–Female	0/9
Female–Male	9/10
Female–Female	16/16
C57Bl/10	
Male–Male	9/9
Male–Female	0/9
Female–Male	10/10
Female–Female	7/7
Female–Male–Male	5/5
Female–Male–Male–Female	4/4

spleen cells from males back to females was successful. As we have never observed successful immunization by transfer of immune spleen cells from male to female, these results indicated that the female immune spleen cells survived in the males during subsequent passage to the second-series males and finally back to the females, and were still sufficient to induce immunity in the female recipients after transfer in the third passage.

DISCUSSION

Immune spleen cells alone from mice with an established immunity to virulent *P. berghei* (VP) could not transfer immunity to these parasites directly. Not even all spleen cells of one donor transferred to a single naive recipient could protect against a low dose challenge with VP. Transferred immune spleen cells, however, conferred protection to IP challenge, and boosting of immune spleen cells with IP generated protection to VP within a week. In addition, more than 10^7 immune spleen cells/mouse and more than 10^4 IP had to be given for optimal results. Additional challenges of the donor with IP shortly before transfer of immune spleen cells were not protective, suggesting that boosting the donor did not provide more efficient immune spleen cells and that interaction between IP and immune spleen cells must take place in the recipient.

Only spleen cells, not lymph node cells, from immune donors could transfer immunity, and recipients only resisted VP challenge if they were stimulated by an IP infection. Neither an IP nor a VP infection alone (even under sulphonamide control) was protective in the 1-week procedure.

The results suggest that IP have, but VP lack, essential information for the stimulation of immune effector cells, and that IP stimulation drives the immune system to protective reactions that subsequently control pathological reactions triggered by VP. Indeed, VP infections tend to trigger immunopathological reactions such as cerebral malaria,[14] and IP do not (manuscript submitted for publication). In view of the essential role of CD4+ T cells in development and maintenance of immunity in the model described here (manuscript submitted for publication), one hypothesis is that IP preferentially trigger a particular T-cell subset, the activity of which controls the stimulation of another subset specifically triggered by VP [e.g. T-helper type-2 (Th2) cells versus Th1]. Cytokine-regulated activity of T-cell subsets[20–23] and activation of T-cell subsets producing either pathology or protection has been described in several parasite–host combinations,[24–26] including *Plasmodium* infections in mice.[27,28]

The observations that not even chemotherapeutically controlled VP stimulation of immune spleen cells in recipient mice can induce immunity, but that either IP alone or in combination with VP can (Table 1), suggest that VP lack essential stimulatory information, which may make them a poor source of vaccination targets except those that aim at anti-disease protection.[29]

Successful immunization by transfer not only depends on the presence of immune spleen cells and IP, but IP must proliferate and stimulate transferred cells for a period of approximately 1 week to induce VP protection (Table 1). The observation that extra IP challenges of donors immediately before transfer of immune spleen cells did not protect recipient mice to VP challenge supports this notion. The reason why IP must proliferate for a period of 1 week is not clear. One possibility is that the immunizing principle is released by IP rather than being a surface molecule. Another possibility is that only live proliferating IP arrive at places where cells of the immune system can be stimulated (e.g. in the white pulp of the spleen). Still another possibility is that only proliferating IP (not VP) can induce an essential architectural change in the spleen, such as like the barrier cell system described by Weiss.[3]

The observation that female immune spleen cells could be transferred to two series of male recipients and that immune spleen cells from the last male induced immunity in subsequent female recipients, suggests that the original female cells survived through two male passages and were still sufficient for subsequent transfer of immunity to females. This supports a role for specifically triggered immune spleen cells in the development of protection.

In the work reported in this paper, immunity to IP, but not to VP, was transferred directly by immune spleen cells, which may suggest that only memory to IP was present.

There was a clear association between development of immunity after transfer and production of anti-parasitic antibody. It cannot be excluded, however, that antibody levels parallel rather than precede development of immunity. If antibody is important, this may indicate that Th2 activity is important in this model. If protective or pathology-inducing Th responses can later be demonstrated, then it will be important to identify antigens that mediate these protective or pathological outcomes.

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